

Screening of the Ethnobotanicals against MDA-MB-231 and MCF-7 breast cancer cell lines

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Abstract

The present study aims to investigate the therapeutic properties of a library of the medicinal plants for their potential anticancer activity against breast cancer cells. The viability of MDA-MB-231 and MCF-7 breast cancer cells was determined by MTT and trypan blue exclusion assays. The cellular cytotoxicity and levels of cytosolic enzyme, Lactate Dehydrogenase (LDH) were measured by assessing μ moles of NADH/well/min. Additionally DNA laddering and FACS analysis was done to further investigate the nature of cell death in these cells. Out of the tested plants, only *Abrus precatorius* L. extract showed a very significant cytotoxic activity on both cell lines. Trypan-blue assay showed the cell viability was significantly decreased in a dose dependant manner in both cell lines after 48 h of treatment. On the other hand, LDH activities in the cultured media were significantly elevated in both cell lines in a dose dependant manner as compared to the control. A prominent DNA laddering pattern in agarose gel was observed, which is an important hallmark of apoptosis. FACS analysis confirmed the apoptosis by the appearance of Sub G0/G1 peak in treated cells as compared to the control. This analysis will facilitate the use of traditional medicine in cancer treatment.

Keywords: Ethanobotany, *Abrus precatorius* L., Breast cancer, Cytotoxicity.

1. Introduction

The search for cancer treatment continues to be a global effort. As part of this effort, many natural products have been tested against various types of cancer cell lines¹ and still there has been a long standing interest in the identification of medicinal plants and derived natural products for developing cancer therapeutics.² India has one of the richest plant medical traditions in the world and around 25,000 effective plant-based formulations used in ethanobotanical communities in India. But only a small portion has been explored phytochemically.³ Therefore, the search for alternative drugs that are both effective and non-toxic in the treatment of cancers is an important research line.⁴ In fact, increased efforts are being made to isolate the bioactive products from medicinal plants for their possible utility in cancer treatment.⁵ The National Cancer Institute (NCI) alone has reportedly screened more than 120,000 plant extracts from 35,000 species for isolation and characterization of novel anticancer phytochemicals.^{6,7} Hartwell, in his review of plants used against cancer, lists more than 3000 plant species that have reportedly been used in the treatment of cancer.⁸ Moreover, from 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively derived from plants and there are a noteworthy number of drugs that were obtained by molecular modification of natural products.⁹ Hence, for some decades traditionally used plants are gaining more attention in searching of bioactive compounds with described properties.¹⁰ A focused research will not only achieve definitive knowledge about the plant and its biological properties but also it may facilitate the synthesis of more potent drugs with reduced toxicity and better efficacy.¹¹ Subsequently there is always a need to screen a number of plants with medicinal value for promising biological activity.¹² To make best use of traditional knowledge about anticancer properties of medicinal plants a systematic study is required which involves (1) Screening of medicinal plants known for anticancer property. (2) Selection of one among them possessing promising anticancer activity. (3) Bioassay guided isolation and identification of active principles as well as subsequent determination of the spectra and potency of the isolated compounds.¹³ Hence, the present study aims to investigate the therapeutic properties of a library of the selected medicinal plants (Table 1) for their potential anticancer (antiproliferative and cytotoxic) activity against human breast cancer cell lines viz., MDA-MB-231 and MCF-7. The selection of medicinal plants for screening was based on their ethnobotanical uses as well as reported anticancer properties in the literature.

2. Materials and Methods

2.1 Plant material

Fresh, disease free leaves of 12 medicinal plants were collected from Danvantrivanna, Bangalore University campus, Bangalore, Karnataka, India (Table 1). The plant material was identified and authenticated by an expert taxonomist. The collected plant material was washed thoroughly under the running tap water in order to remove dirt, germs and other contaminants, shade dried then powdered and used for extraction.

Authenticated voucher specimens of the plants are deposited in the herbarium of Molecular Diagnostic and Nanobiotechnology Laboratories, Department of Microbiology and Biotechnology, Bangalore University, Bangalore for future reference¹⁴.

Table 1: Medicinal plants screened against MDA-MB-231 and MCF-7 breast cancer cell lines for anticancer activity.

Sl. No.	Plant Species	Family	Common Name	Voucher number
1.	<i>Abrus precatorius</i> L.	Fabaceae	Indian Liquorice	BU/MKS-MDL/AP-1
2.	<i>Bauhinia variegata</i> L.	Fabaceae	Kachnar	BU/MKS-MDL/BV-2
3.	<i>Caesal piniagilliesii</i>	Fabaceae	Bird of Paradise	BU/MKS-MDL/CG-3
4.	<i>Decale pishamiltonii</i> W.	Apocynaceae	Swallow root	BU/MKS-MDL/DH-4
5.	<i>Emblca officinalis</i> L.	Phyllanthaceae	Amla	BU/MKS-MDL/EO-5
6.	<i>Jatropha gossypifolia</i> L.	Euphorbiaceae	Physic nut	BU/MKS-MDL/JG-6
7.	<i>Moringa oleifera</i> L.	Moringaceae	Moringa	BU/MKS-MDL/MO-7
8.	<i>Plumbago zeylanica</i> L.	Plumbaginaceae	Plumbago	BU/MKS-MDL/PZ-8
9.	<i>Salacia oblonga</i> L.	Celastaceae	Saptrangi	BU/MKS-MDL/SO-9
10.	<i>Tinospora cordifolia</i> M.	Menispermaceae	Guduchi	BU/MKS-MDL/TC-10
11.	<i>Vitex negundo</i> L.	Verbenaceae	Huang Ping	BU/MKS-MDL/VN-11
12.	<i>Ziziphus jujuba</i> Mill.	Rhamnaceae	Jujube	BU/MKS-MDL/ZJ-12

2.2 Preparation of Plant Crude Extracts

Aqueous leaf extracts of 12 medicinal plants were prepared by the process of maceration.¹⁵ Dried plant sample was grinded to fine powder in mixer-grinder and sieved. The powder of each sample was suspended in double distilled water at the rate of 10 gms in 60 ml, sonicated for 15 minutes and mixed with magnetic stirrer for overnight. Each extract was passed through two layered cheese cloth.¹⁶ The filtrate was then centrifuged at 5,000 rpm for 30 min. Lyophilization of the mother solution was done with a Speedvac (Savant Speedvac SC100 Centrifugal Evaporator, USA). Lyophilized aqueous extracts were dissolved in PBS (sterile) to a stock concentration of 50 mg/ml and then passed through a 0.2 µm filter (Sartorius Stedim) for sterilization and was then used for cytotoxicity assays.

2.3 Cell Lines and Culturing of cells

Human breast cancer cell lines MDA-MB-231 and MCF-7 cells were procured from Indian Institute of Science (IISc), Bangalore (India). Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Once the cells had covered about 80% of the surface, they were trypsinized and the titer was adjusted using haemocytometer for antiproliferative and cytotoxicity measurements.¹⁷

2.4 Analyses of Cell viability of Selected Medicinal Plants by MTT Assay

To evaluate *in vitro* antiproliferative and/or cytotoxic activity of 12 medicinal plants listed in table 1, standard colorimetric MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay was done.¹⁸ MDA-MB-231 and MCF-7 cells (5x10³ cells/well) were seeded to 96-well microtiter plates (Falcon, Becton Dickinson, NJ). After 24 hours of plating, cells were serum starved for 24 hours. Respective concentrations of all extracts were added in serum free medium and 0.2 % (v/v) DMSO (Merck Germany) as negative control. After 48 h of treatment, 50 µl of MTT (2 µg/ml) reagent (Sigma, St. Louis, MO), was added to each well. The plates were incubated at 37°C in humidified atmosphere with 5% CO₂ for 4 h. Medium was removed and 200 µl DMSO was added.¹⁹ The absorbance at 595 nm was measured using a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc. USA). The percentage of cell viability was calculated according to the following equation.²⁰ Percentage of cell viability = (OD of treated cells / OD of control cells) × 100. The concentration of drug that inhibits 50% of the cells (IC₅₀ values) for these samples were obtained from dose-response curves.

2.5. Trypan Blue Exclusion Assay for Cell Viability

For the determination of cell viability, MDA-MB-231 and MCF-7 breast cancer cells were plated at a density of (1 × 10⁶ cells/well) in 24 well tissue culture plate and cultured for 48 hours at 37°C. The medium was replaced with serum-free medium and the cells were treated with various concentrations of *A. precatorius* leaf extract (50, 100, 200, 500 and 750 µg/mL) for a further 48 hours. The cultures were harvested and washed twice with PBS, the cell pellet was then suspended with 0.5 ml PBS. Then 20 µl of cell was mixed with equal volume of 0.4 % trypan blue (Sigma-Aldrich, USA). Cells with an intact membrane are able to exclude the dye, while membrane damaged cells after exposure with extract uptake trypan blue and become blue cells.²¹ Number of live and dead cells was directly counted on hemocytometer under inverted microscope. The percentage viability for the cells was calculated as (live cells/total cells) x100. The calculated percentage of unstained cells will represent the percentage of viable cells.

2.6. Lactate dehydrogenase leakage assay

Cytotoxicity induced by leaf extract of *Abrusprecatorius* was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. LDH, a cytoplasmic enzyme, release is the consequence of cell membrane rupture.²² Cell membrane rupture was defined as the ratio of LDH activity in the supernatant of treated cells to the LDH activity released in the control cells. MDA-MB-231 and MCF-7 breast cancer cells (1 × 10⁶ cells/well) were plated in 100 µL of medium/well in 96-well plates and were allowed to attach to the plate for 24 h. After cell attachment (24 h) cells were treated with increasing concentrations of *A. precatorius* leaf extract (50, 100, 200, 500 and 750 µg/mL). The extracellular LDH activity was measured in the medium after 24 h. Following treatment, the culture medium was aspirated and centrifuged at 3000 rpm for 5 min in order to obtain a cell free supernatant. The activity of LDH in the medium was determined using 100 µL of cell free supernatant of the control and *A. precatorius* treated MDA-MB-231 and MCF-7 breast cancer cells was added to 1-mL cuvette containing 0.9mL of a reaction mixture to yield a final concentration of 1mmol/L pyruvate, 0.15 mmol/L NADH and 104 mmol/L phosphate buffer, pH 7.4. After thoroughly mixed, the absorbance of the solution was measured at 340 nm for 45 s. LDH activity was expressed as µ moles of NADH used per minute per well. All experiments were repeated three times in triplicates.

2.7. DNA Laddering Assay

MDA-MB-231 and MCF-7 breast cancer cells were treated with aqueous extract of *A.precatorius* for 12 h. At the end of treatment, both adherent cells and floating cells were harvested and then treated with 100 µl lysis buffer (1% NP-40 in 20 mM EDTA, 50 mMTris-HCl, pH 7.5). The supernatant was collected by centrifugation for 5 min at 1600 g. The supernatant was brought to 1% SDS and treated for 2 h with RNase-A (final concentration 5 mg/ml) at 56°C followed by digestion with proteinase K (final concentration 2.5 mg/ml) for at least 2 h at 37°C.²³ After addition of 1/2 volume 10 M ammonium acetate, the DNA was precipitated with 2.5 volume cold absolute ethanol. The DNA pellet was obtained by centrifugation at 12,000 g for 20 min at 4°C, and the DNA was dissolved in TE buffer (10 mMTris-HCl, pH 8.0, 1 mM EDTA). Purity and quantification of the DNA were done by measuring absorbance at 280 nm. Samples (100 ng each) were mixed with loading dye (xylene cyanol in 30% glycerol) and resolved at 80V 2 h on a 1.5% agarose gel containing 0.5 mg/ml ethidium bromide. A 100 bp ladder DNA

sample was used as a marker. The resulting DNA fragmentation was visualized under UV transilluminator (UVI Pro) followed by Polaroid photography in gel documentation unit.

2.8 Cell Cycle Analysis

Cell cycle analysis was performed by propidium iodide (PI) based measurements of DNA content of the cells by flow cytometry. MDA-MB-231 and MCF-7 breast cancer cells were plated in a six well plate at the rate of 4×10^5 cells per ml and incubated for 24 hours. Both the cells lines were treated with aqueous extract of *A. precatorius* for another 24 hours. After treatment, the cells were trypsinized, washed with DPBS and fixed with 70% cold methanol and the mixture kept in -20°C overnight. After centrifugation for 5 min at 2000rpm at 4°C , the pellet was treated with 2mg/ml RNase-A at 37°C and stained with 50 $\mu\text{g/ml}$ propidium iodide.²⁴ The sample was then subjected to FACS analysis and the percentage of cells in each stage of the cell cycle was determined using Cell-Quest software (Becton–Dickinson Biosciences, USA).

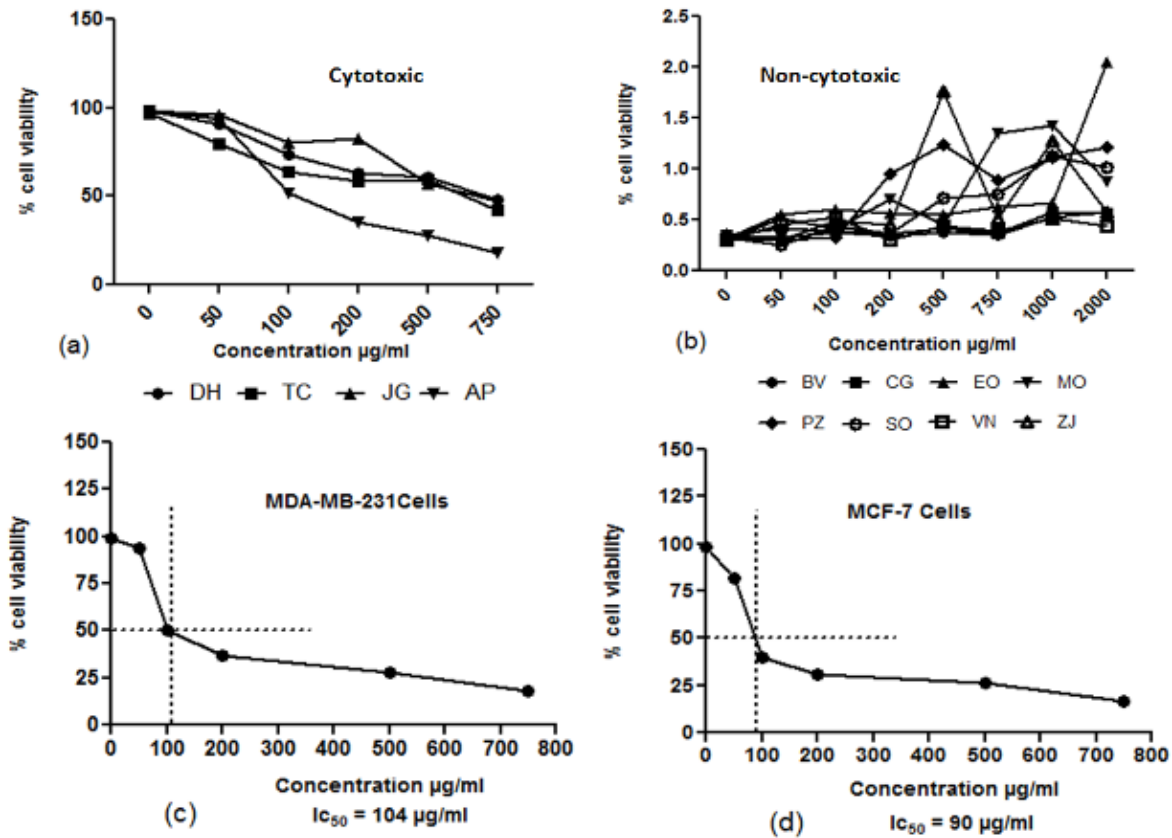
2.9 Statistical Analysis

Results were expressed as means \pm SD of replicates. Comparison between data sets was performed using one way analysis of variance (ANOVA) followed by Student's t-test. Differences were accepted as statistically significant at $p < 0.05$.

3. Results

3.1 Cytotoxicity effects of an aqueous leaf extracts of medicinal plants on breast cancer cell proliferation

Figure 1: Cytotoxic effects of plants extracts



MTT assay of extracts (a) showing cytotoxic activity (DH-*Decalepis hamiltonii*, TC- *Tinospora cordifolia*, JG-*Jatropha gossypifolia*, AP- *Abrus precatorius*), (b) without cytotoxic activity after 48 h treatment on MDA-MB-231 breast cancer cells (BV-*Bauhinia variegata*, CG-*Caesalpinia gilliesii*, EO- *Emblica officinalis*, MO-*Moringa oleifera*, PZ-*Plumbago zeylanica*, SO- *Salacia oblonga*, VN- *Vitex negundo*, ZJ- *Ziziphus jujube*). (c). IC_{50} values of *A. precatorius* extract on MDA-MB-231 and (d) MCF-7 cell lines.

3.2 Morphological Studies of MDA-MB-231 and MCF-7 Cells before and after Treatment

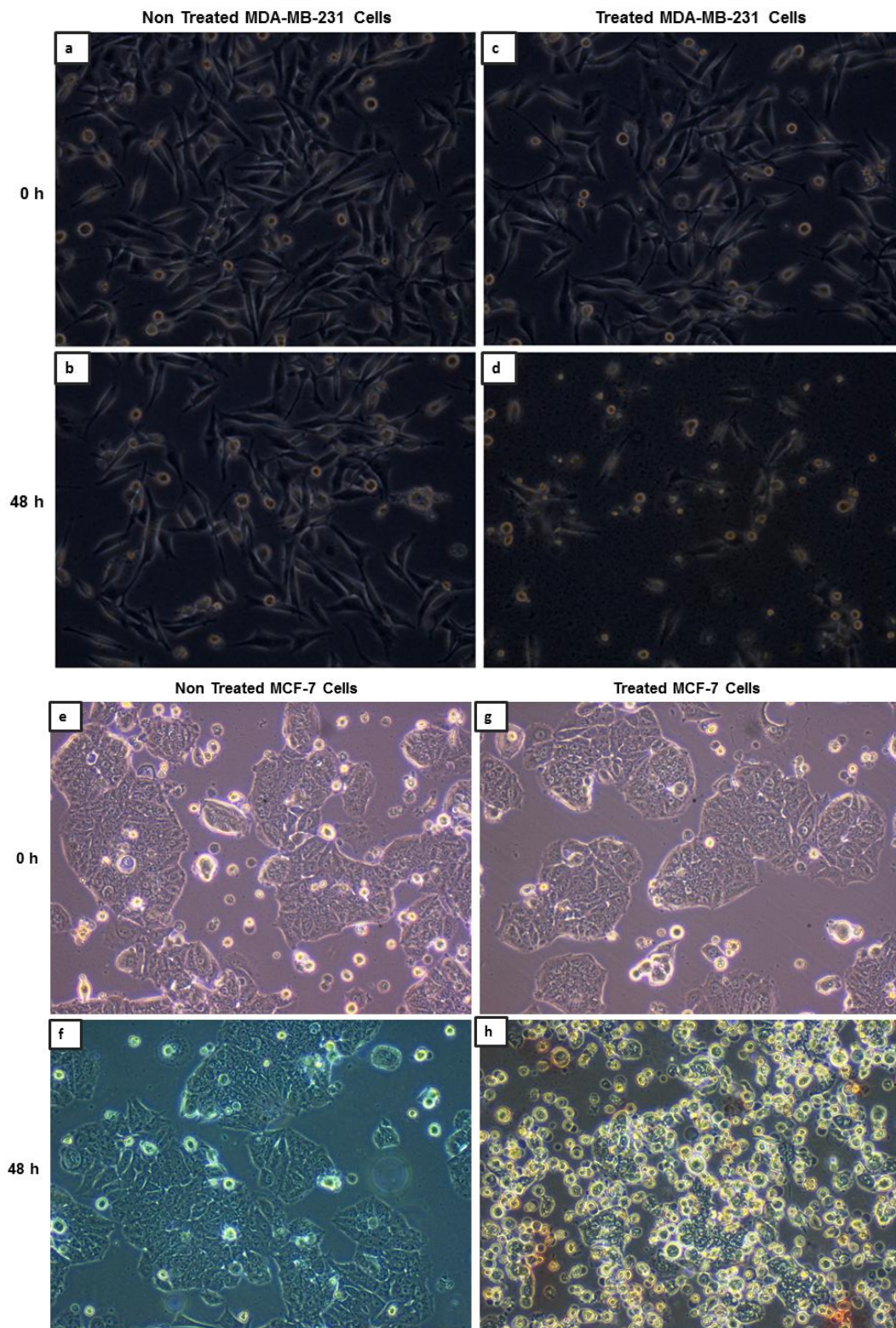


Figure 2: Microscopic view of MDA-MB-231 and MCF-7 cells before(a,b and e,f) and after (c,d and g,h) treatment with *A. precatorius* extract at IC_{50} values in 0 h and 48 h with magnification of 10X.

3.3. Assessing cellular viability by Trypan blue exclusion Assay

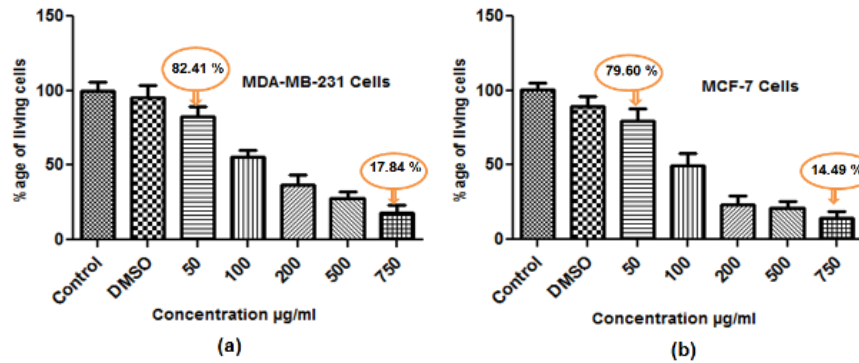


Figure 3: Effect of leaf extract of *A. precatorius* on cell viability of (a) MDA-MB-231 cells (b) MCF-7 cells in a dose dependent manner as measured by Trypan Blue Assay. Viability decreased from 82.41% and reached a minimum value of 17.84% in MDA-MB-231 cells and in MCF-7 cells, viability decreased from 79.60% to 14.49% at 50 and 750 µg/ml respectively.

3.4. Effects of *A. precatorius* on Lactate Dehydrogenase (LDH) Activity

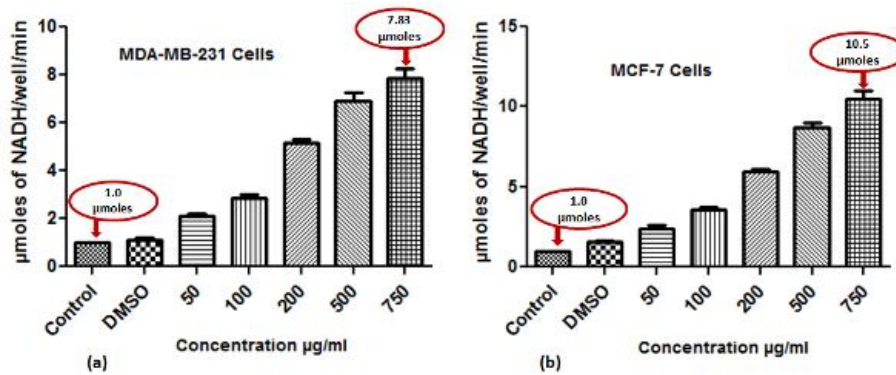


Figure 4: Effect of leaf extract of *A. precatorius* on the levels of cytosolic enzyme LDH released into the medium of the control and *A. precatorius*-treated (a) MDA-MB-231 cells (b) MCF-7 cells in a dose dependent manner. LDH level was significantly elevated from 1.0 µmoles to 7.83 µmoles in MDA-MB-231 cells and in MCF-7 cells was increased from 1.0 µmoles to 10.5 µmoles at 750 µg/ml of NADH/well/min.

3.5 *A. Precatorius* induces significant DNA fragmentation in MDA-MB-231 and MCF-7 cells

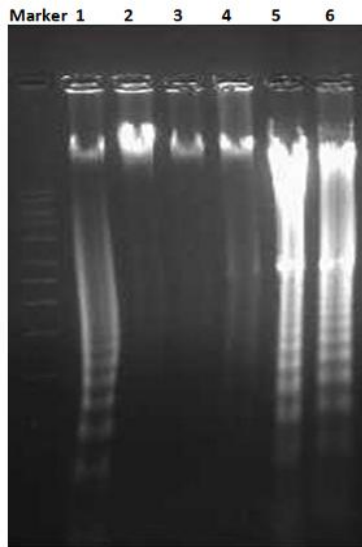


Figure 5: Agarose gel electrophoresis displaying DNA fragmentation in MDA-MB-231 and MCF-7 cells incubated with *A. precatorius* extract at 104 and 90 µg/ml respectively. Marker 100 bp; Lane-1: positive control; 2: negative control; 3: Untreated MDA-MB-231; 4: Untreated MCF-7; 5: Treated MDA-MB-231; 6: Treated MCF-7 cells.

3.6 A. *Precatorius* induces apoptosis as revealed by FACS analysis

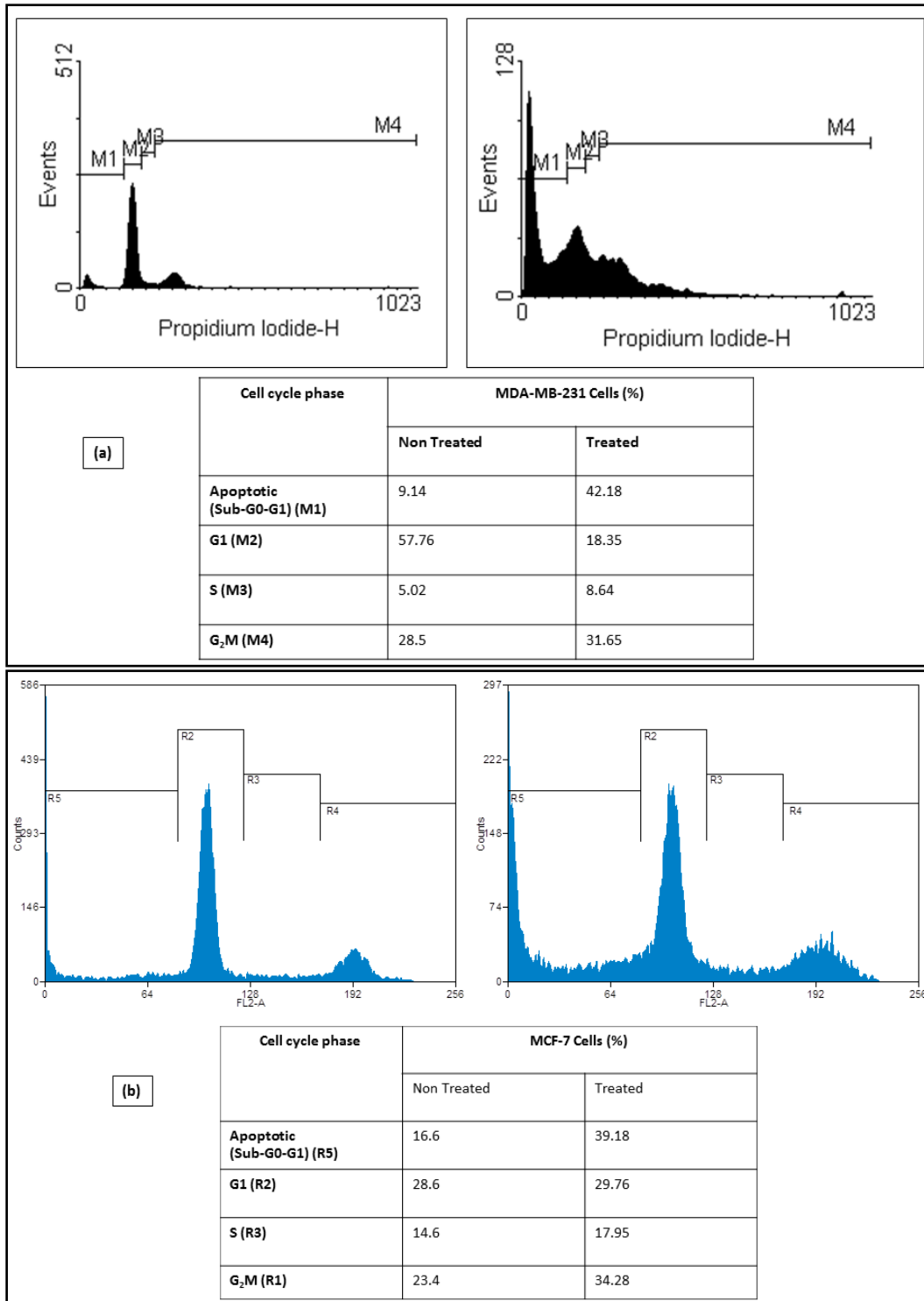


Figure 6: Flow cytometric analysis of the DNA histograms of PI-stained MDA-MB-231(a) and MCF-7 cells (b) treated with *A. precatorius* at its respective IC₅₀ concentration. The percentages of cells in the indicated phases of the cell cycle are mentioned. Sub-G0/G1 peak is an indicative of apoptotic cells in both the cell lines.

4. Discussion

Due to the high mortality rates of the cancer and the lack of efficient chemotherapy, there is a continued necessity for new alternatives for treatment and prevention of cancer²⁵. Plant derived products are considered excellent sources for the discovery and development of novel cancer chemoprotective and chemotherapeutic drugs²⁶. There are two main strategies for the selection of plants species in anticancer drug discovery: random screening and ethno-medical knowledge²⁷. In the present study based on the ethno-botanical and ethno-pharmacological data,

twelve traditional Indian medicinal plants were selected for their potential anticancer (antiproliferative and cytotoxic) activity against breast cancer cells. Cytotoxicity screening models provide important preliminary data to help select plant extracts with potential anticancer properties²⁸.

In the present study cytotoxicity was determined using the standard colorimetric MTT assay. Based on this assay out of the selected medicinal plants, only *A. precatorius* showed significant activity on growth and proliferation of breast cancer cells. Previous preliminary studies also reported significant cytotoxic activities of various plant crude extracts on various types of breast cancer cell lines (Junior *et al.*, 2010). Microscopic examination shows a significant morphological changes, such as shrinkage and detachment of cells from the surface in both cell lines, treated as compared to non-treated cells. Further, trypan blue exclusion assay method showed the reduction of cell viability in dose dependent manner in treated MDA-MB-231 cells as compared to control cells. Cytotoxicity induced by leaf extract of *A. precatorius* was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. LDH, a cytoplasmic enzyme, release is the consequence of cell membrane rupture. Cell membrane rupture was defined as the ratio of LDH activity in the supernatant of treated cells to the LDH activity released in the control cells. The levels of cytosolic enzyme Lactate Dehydrogenase (LDH) was significantly elevated after 48 h of *A. precatorius* leaf extract treatment in the medium when compared to the control. One of the important hallmarks of apoptosis is DNA fragmentation. As shown in agarose gel electrophoresis, increased DNA fragmentation was apparent in treated MDA-MB-231 and MCF-7 cells as compared to the untreated cells. Flow cytometric analysis showed that *A. precatorius* has a pro-apoptotic activity on both MDA-MB-231 and MCF-7 cells. A significant increase sub G0/G1 population of cells was analyzed from (9.14% to 42.18%) in MDA-MB-231 cells and (16.6% to 39.18%) in MCF-7 cells after 24 h treatment, as compared to control cells. This is an important indicator of apoptosis.

This study provides an important basis for further investigation into the isolation, identification, characterization and molecular mechanism behind the specific cytotoxic activity of bioactive anticancer phytochemicals present in *A. precatorius* and to emphasize the use of traditional medicine in cancer treatment.

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